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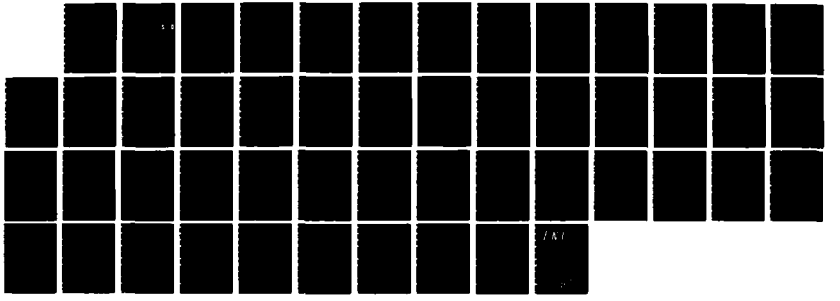

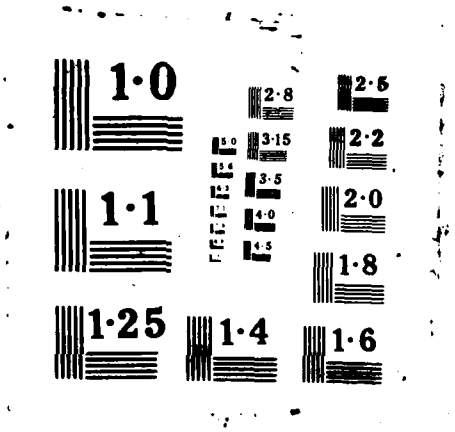
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SELECTIVE TARGETING OF ANTIVIRAL AND IMMUNOMODULATING AGENTS IN THE TREATMENT OF ARENAVIRUS INFECTIONS

Annual Summary Report

J. DAVID GANGEMI

June 30, 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

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University of South Carolina
School of Medicine
Columbia, South Carolina 29208

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The therapeutic value of large negatively charged liposomes as vehicles for the delivery of antiviral or immunostimulatory agents to the lungs of mice infected with herpes simplex (HSV-1) or influenza virus was examined. Both liposome encapsulated and non-encapsulated ribavirin or muramyl tripeptide were compared for their ability to: i) deposite in pulmonary tissue following intravenous administration; ii) reduce virus replication in primary target organs; and iii) protect against lethal virus infections. Liposome carriers provided better lung delivery than did non-encapsulated drug. Moreover, liposome encapsulated MTP (L-MTP) was superior to free MTP in activating alveolar macrophage functions as determined by in vitro functional analysis (e.g. phagocytosis, microbicidal and tumoricidal activities), and in protecting mice against intranasal challenge with 10 LD_{50} of HSV-1. Similarly, intravenous administration of liposome encapsulated ribavirin (L-RIB) (3 mg/mouse) several hours after infection was more effective than free RIB (10 mg/mouse) in protecting mice against intranasal challenge with

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10 LD_{50} of influenza virus. Neither L-RIB nor free RIB was effective in treating HSV-1 infections even though HSV is susceptible to this antiviral agent *in vitro* (MIC_{50}=30ug/ml). However, combination therapy using both L-RIB and L-MTP was more effective than either agent alone in protecting mice from lethal HSV-1 infection. The data suggest that liposomes can enhance the therapeutic potential of both antiviral and immunostimulating agents and are potentially quite useful as vehicles for the targeted delivery of these drugs in the therapy of human viral diseases.

keywords

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SUMMARY

An important prerequisite for the therapeutic success with an antiviral agent is the ability to reach specific targets within the host and be maintained at therapeutic levels without resulting in host toxicity. Drug carries such as liposomes may be useful in this regard since these vehicles can be designed to deliver chemotherapeutic agents to infected target sites and thus avoid exposure of uninfected cells or organs. The data presented in this report was generated during the first year of a three year study designed to examine the potential use of liposomes as carriers for the selective targeting of antiviral and immunomodulating agents in the treatment of viral infections. We have examined the therapeutic value of large negatively charged liposomes as vehicles for the delivery of the broad spectrum antiviral, ribavirin, or the synthetic immunostimulant, muramyl tripeptide, to the lungs of mice infected with herpes simplex (HSV-1) or influenza virus. Both liposome encapsulated and non-encapsulated drugs were compared for their ability to: i) deposite in pulmonary tissue following intravenous administration; ii) reduce virus replication in primary target organs; and iii) protect against lethal virus infections.

Tissue distribution studies using ^{14}C-ribavirin as a marker revealed that liposome carriers resulted in better (5-fold greater) lung delivery than did nonencapsulated drug following intravenous administration. The greater lung concentrations attained by liposome encapsulation was due in part to the selective localization of these carriers in pulmonay macrophages. Moreover, liposome encapsulated MTP (L-MTP) was superior to free MTP (100 ug/mouse) in activating alveolar macrophage functions as determined by *in vitro* functional analysis (eg. phagocytosis, microbicidal and tumoricidal activities), and in protecting mice against intranasal challenge with 10LD$_{50}$ of HSV-1. Similarly, intravenous administration of liposome encapsulated ribavirin (L-RIB) (3 mg/mouse) several hours after infection was more effective than free RIB (10mg/mouse) in protecting mice against intranasal challenge with 10LD$_{50}$ of influenza virus. Neither L-RIB nor free RIB was effective in treating HSV-1 infections even though HSV is susceptible to this antiviral agent *in vitro* (MIC$_{50}$=30ug/ml). However, combination therapy in which both L-RIB and L-MTP were used was more effective than either agent alone in protecting mice from lethal HSV-1 infection.

The data obtained during the initial period of this study has resulted in the following conclusions:

1. The selective tissue localization and retention characteristics provided by liposomes enhance the therapeutic index of antiviral agents such as ribavirin.

2. Liposomes provide a distinctive therapeutic advantage when combination chemotherapeutic strategies are employed by providing the simultaneous delivery of two or more drugs to the same site(s).

3. Because of their natural predilection for the reticuloendothelial system, liposomes provide a highly selective means by which immunostimulants are delivered to macrophages and thereby augment non-specific immunity to virus infections.

FOREWORD

Citations of commericial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. PROBLEM UNDER INVESTIGATION

A number of clinically proven antivirals, such as amantadine, iododeoxyuridine, adenine arabinoside, and acycloguanosine are currently in use; however, to the best of our knowledge, these drugs are of little value in the treatment of arenavirus infections. Several potentially useful antivirals (e.g., ribavirin, and selenazole) possess a broad spectrum of activity against both DNA and RNA viruses (including arenaviruses) and are currently in the investigational stage. Clinical acceptance of these antivirals could be hastened with the development of delivery vehicles which provide sustained release of drug in virus-infected target organs and avoid contact with non-infected tissues.

This study addresses the therapeutic value of liposomes for the targeted delivery of antiviral and immunomodulating agents to infected organs in a diseased host. More specifically liposomes have been examined for their ability to:

1) deliver immunostimulants and antivirals to the site(s) of primary virus replication, enhance cellular immunity, and reduce virus burdens;
2) protect antiviral substances from the normal degradative and clearance mechanisms of the host during the journey from the site of inoculation to the site(s) of infection;
3) increase uptake and retention of antivirals by infected tissues;
4) reduce therapeutic dosages normally required to inhibit virus replication in infected organ and cellular sites; and
5) reduce the toxic effects observed with conventional modes of antiviral therapy.

II. BACKGROUND

Successful drug use in biology and medicine is often prejudiced by the failure of drugs that are otherwise active *in vitro* to act as efficiently *in vivo*. This is because in the living animal drugs must, as a rule, bypass or traverse organs, membranes, cells and molecules that stand between the site of administration and the site of action. In practice, however, drugs can be toxic to normal tissues, have limited or no access to the target and be prematurely excreted or inactivated. A number of antiviral agents (e.g., ribavirin, adenine arabinoside, phosphonacetic acid) have been developed which are highly effective *in vitro* in preventing virus replication and/or cell death; however, their systemic use in man is limited by the induction of toxic effects which occur at dosages required to maintain effective drug concentrations in the infected organ. In particular, sustained treatment often results in leukopenia and subsequent immunosuppression which may affect the outcome of treatment since recovery from most viral infections also involves the cooperation of host immune responses. As in the case of ribavirin where dose limiting toxicity is the development of anemia (1,2), there is now growing optimism that such problems may be resolved with the use of carrier vehicles that will not only protect the nontarget environment from the drugs they carry but also deliver and facilitate their release at the site(s) in which they are needed.

During the past decade, a variety of carrier types have been advocated for the selective targeting of antitumor drugs. Thus, there are numerous reports on the association of drugs such as anthracyclines, methotrexate, bleomycin, chlorambicin, and 1-B-D-arabinofuranosyl cytosine (cytosine arabinoside) with carriers such as DNA (3,4), liposomes (5,6), immunoglobulins (7,8), hormones (9,10), red blood cell ghosts (11) and other proteins (12,13) or polypeptides (14). Most of these carriers have the ability to selectively interact with target cell surfaces and are subsequently endocytosed and transferred to the lysosomal compartment. Free drug is released intracellularly when the bond between the drug and the carrier is hydrolysed by lysosomal enzymes (15). In contrast, liposomes may sometimes deliver their contents directly to the cytoplasm following fusion with the target cell membrane (16). This mode of delivery is useful for drugs which are susceptible to lysosomal enzymes since membrane fusion is a mechanism through which contact with the lysosomal compartment of the cell can be bypassed.

In addition to the examples already cited, liposomes have also been used as carriers for i) the hepatic delivery of arsenicals in the treatment of leishmaniasis (17), ii)

iododeoxyuridine in the treatment of herpes keratitis (18), and iii) amphotericin B in the treatment of Candida albicans (19), murine leishmaniasis (20), histoplasmosis (21), and cryptococcosis (22). The mechanism(s) by which liposomes enhanced the chemotherapeutic index of these drugs has not been defined; nonetheless, increased and prolonged tissue concentration of both iododeoxyuridine and amphotericin B in infected sites are most likely involved. Our rationale for the use of liposomes as carriers in the delivery of antivirals to virus infected tissues or organs in a diseased host is based on previous observations in which these macromolecular carriers provided a therapeutic advantage. Ribavirin (1-B-D-ribofulanosyl-2, 4-triazole-3-carboxamide; ICN Pharmaseuticals) was selected as the prototype antiviral to be encapsulated because of its broad spectrum activity and relatively low toxicity . The synthetic analog of muramyl dipeptide, muramyl tripeptide-phosphatidyl ethanolamine (MTP-PE; hereafter designated MTP) manufactured by Ciba Geigy was selected as the immunostimulant of choice because of its lipophilic properties, defined immunoenhancing properties, and potential for use in humans.

III. EXPERIMENTAL APPROACH

Our experimental approach during the first year of this three year study was designed with the following goals in mind:

1. preparation and physical/chemical characterization of liposome carriers containing either the broad spectrum antiviral, ribavirin, or the synthetic immunostimulant muramyl tripeptide;

2. examination of the tissue distribution of liposome encapsulated drugs following intravenous inoculation; and

3. examination of the therapeutic effectiveness of liposome encapsulated ribavirin or MTP, (either alone or in combination) in the treatment of both herpesvirus and influenza virus-induced pneumonitis.

A. Liposomes

Multilamellar vesicles (MLV) containing phosphatidyl serine, phosphatidyl choline and cholesterol (3:7:1 ratio) were produced using a rehydration procedure and examined in the cell sorter for size heterogeneity (Figure 1). This type of liposome was selected because of its encapsulation efficiency, deposition in pulmonary tissue following intravenous administration, and ease in manufacturing. Chemical characterization of liposomes was done by high performance liquid chromatography while tissue distribution following intravenous administration was determined using autoradiographic techniques. The prophylactic and therapeutic potential of liposome encapsulated ribavirin or MTP was examined in C3H mice infected intranasally with either herpes or influenza virus (see below). Mice received 1.4 mg phospholipid/mouse/dose either alone (sham liposome) or in combination with MTP or ribavirin.

B. Animal Models

The following is a description of the experimental models of virus-induced disease used to evaluate the therapeutic value of liposome encapsulated antivirals and/or immunostimulants. The C3H/HeN mice utilized in these studies were obtained from our breeding colony maintained at the University of South Carolina School of Medicine. This breeding facility provides for the continuous source of young mice.

Influenza virus Pneumonitis: The virus used in our laboratory is the mouse adapted H3N2 strain of influenza virus (Aichi). When 10 LD_{50} of this viral strain is administered intranasally into six to seven week old C3H/HeN mice, death, due to interstitial pneumonia, occurs in five to seven days.

HSV-1 Pneumonitis: The virus used in our laboratory is a human isolate (VR3 strain) of type 1 herpes simplex virus obtained from Dr. Andre Nahmias (Emory University, Atlanta, GA). Intranasal inoculation of three to five week old C3H/HeN mice with LD_{50} of virus results in a fulminant pneumonitis and adrenalitis, and death occurs between five to eight days following infection. A unique aspect of this model is that encephalitis does not occur. As illustrated in Figure 2, the lungs are the primary target organ and the adrenals a secondary site of virus replication. Examination of the brain and other organs does not reveal consistent levels of virus replication. As indicated in this figure, peak virus titers in the lung occur 48 hours post-infection while mice begin to die one to three days later. Immunoperoxidase staining of lungs and adrenals reveals massive viral antigen accumulation in these organs 48-120 hours after infection.

HSV-1 Encephalitis: The virus used to induce encephalitis is a human isolate (MB strain) of type 1 herpes simplex virus obtained from Dr. Richard Whitley (Univ. Ala, Birmingham, AL). Footpad inoculation of four to five week old C3H/HeN mice results in virus replication in the sciatic nerve, spinal cord and brain, and mice die of encephalitis six to eight days after inoculation. Immunoperoxidase staining of viral antigen has been used to confirm this mode of virus dissemination.

IV. RESULTS

A. Liposome Preparation and Characterization

Liposomes were prepared by the procedures described by Kende et al (23) and Fidler et al (24). As described above, the vehicles we selected in the initial phase of our studies were large, negatively charged multilamellar vesicles composed of phosphatidyl choline and phosphatidyl serine. A small amount of cholesterol (23) was added to ribavirin liposomes to reduce leakage. The source of lipids, preparative procedure, and storage conditions were standardized to avoid possible variation in the efficiency of drug encapsulation or particle size. The size distribution of liposome preparations containing either ribavirin or MTP was determined using a fluorescent activated cell sorter. The characteristic light scatter profiles of these carriers were used to determine the distribution of particle sizes (Figure 1). Approximately 70% of the liposome encapsulated ribavirin particles produced by our standardized procedures are 1-2 microns, 15% are 2-5 microns, 10% are 5-10 microns, and 5% 10 microns or larger, the later are aggregates. Very similar size distributions appear in MTP liposome preparations. We have recently initiated the production of small (20 nanometer) unilamellar liposomes using a commericially available instrument (Liposomat). Aerosols containing small liposomes will be prepared and their therapeutic potential examined during the second year of our studies.

Quantitative analysis of ribavirin encapsulated into liposomes was done using radiolabeled drug, and as illustrated in Table 1, an encapsulation efficiency of approximately 20% was observed. A high performance liquid chromatography (HPLC) procedure is being developed by one of our co-investigators, Dr. Carl Bauguess, to quantitate precise levels of ribavirin and MTP per mole of lipid present in liposome carriers. HPLC assays for the determination of cell associated liposome encapsulated ribavirin or MTP are also being developed.

B. MTP Induced Augmentation of Peritoneal and Alveolar Macrophage Functions

A number of experiments were designed to assess the activation of peritoneal and alveolar macrophages following single or multiple doses of MTP. Figure 3 illustrates the inflammatory cell response observed in the peritoneal cavity following a single intraperitoneal (i.p.) inoculation with 100 ug of free or liposome encapsulated MTP (MTPLIP). Note that the cell response following inoculation with liposome encapsulated MTP preceeded that observed with the classical

immunostimulant, C. parvum, by several days. Many of the inflammatory cells observed 48 hours post MTP (100 ug/mouse) inoculation consisted of macrophages (Figure 4).

Kinetic analysis of peritoneal macrophage phagocytosis is presented in Figure 5. As illustrated, phagocytosis of radiolabeled (Cr^{51}) opsonized sheep red blood cells was most active two days after a single i.p. inoculation with either free or liposome encapsulated MTP (100 ug/mouse). Liposome encapsulated MTP was more effective than free MTP in augmenting the Fc mediated phagocytic activity of these cells. Moreover, peak activation occurred several days prior to that observed with the classical immunostimulant, C. parvum.

The effects of multiple i.p. doses of MTP on inflammatory responses in the peritoneum are illustrated in Figure 6. Multiple MTP doses were no better than a single dose in increasing the number of inflammatory cells. Likewise multiple i.p. doses did not further enhance or keep the phagocytic activity of peritoneal macrophages elevated longer than that observed with a single dose (Figure 7).

The ability of MTP to activate alveolar macrophage functions was also examined. Mice treated intravenously with 100 ug of MTP were given intranasal inoculations of 1 x 10^9 fluorescein-labeled Staphylococcus aureus organisms. One hour after S. aureus administration alveolar macrophages were harvested by transtracheal lavage and examined with the cell sorter. Figures 8 and 9 illustrate the analytical profiles obtained when the phagocytic alveolar macrophages containing fluorescent S. aureus organisms are examined by flow cytometry. Peak movement to the right of the horizontal scale indicates more vigorous phagocytosis and provides an indication of the number of phagocytic cells as well as the degree to which they are phagocytosing S. aureus in vivo. As illustrated in Figure 8 liposome encapsulated MTP (MTLIP) was a more effective alveolar macrophage activator than either sham liposome (SHLIP) or free MTP (Fig. 9). Likewise the intravenous or intraperitoneal inoculation of liposome encapsulated MTP is superior to free MTP in augmenting the ability of both alveolar and peritoneal macrophages to kill phagocytosed S. aureus organisms (Figure 10). Moreover preliminary studies indicate that liposome encapsulated MTP is more effective than free MTP in the activation of pulmonary macrophage tumoricidal activity (data not shown).

C. Use of Liposome Encapsulated Ribavirin and MTP in the Treatment of Viral-Induced Pneumonitis

We selected large negatively charged multilamellar liposomes as carrier vehicles since previous observations (24) suggest that it is possible to alter tissue distribution and/or persistence of encapsulated drugs.

Moreover, it is possible to target drug to infected organs without exposing nontarget cells or organs. Figures 11 and 12 illustrate the tissue distribution of both nonencapsulated and liposome encapsulated radiolabeled ribavirin following intravenous inoculation. Note that free drug rapidly deposits in the liver and is cleared by 24 hours. In contrast, encapsulated drug accumulates in the liver, spleen and lung and persists in these organs over the twenty-four hour observation period. Moreover, autoradiographic data revealed a selective cellular localization in pulmonary and splenic macrophages. The distribution profile of liposome encapsulated ribavirin suggests that this method of delivery should be particularly useful in virus infections of the lung and liver.

To examine the efficacy of liposome encapsulated vs free ribavirin in the treatment of pulmonary infections, mice were given ribavirin several hours after intranasal challenge with influenza or HSV-1. Figure 13 illustrates the therapeutic value (prolongation of mean time to death) of ribavirin in either free or encapsulated form. Note that liposome encapsulated ribavirin at 3 mg per mouse per dose was more effective than free drug given at 10 mg per mouse per dose in preventing mortality from influenza infection. Table 2 shows that the survivors in the encapsulated ribavirin group all had elevated antiviral antibody titers and were resistant to rechallenge with 100 LD_{50} of virus. In contrast, similar dosages of free or liposome encapsulated ribavirin was not effective in the HSV-1 model of pneumonitis (Figure 14).

We also examined the therapeutic activity of free and liposome encapsulated MTP in the treatment of HSV-1 pneumonitis. Muramyl tripeptide is a derivative of MDP which contains an additional alanine and phosphatidyl ethanolamine residue. Addition of the phospholipid to MDP provides lipophilic characteristics and thus allows for stable integration of this molecule into liposome lamellae. When MTP was given several days before, on the day of and several days after intranasal challenge with virus, encapsulated MTP was more effective than free drug in limiting mortality (Figure 15). In addition, survivors of MTP treatment had elevated levels of serum neutralizing antibodies and were resistant to rechallenge with 100 LD_{50} of virus (Table 3). While two or three doses of MTP were effective, a single dose given prophylactically was ineffective (data not shown). Both free and liposome encapsulated MTP treatments resulted in reduced virus levels in lungs and adrenals of infected mice (Figures 16 and 17, respectively) and no infectious virus was recovered from the blood of treated and infected animals (Table 4). In Contrast no therapeutic effect of either free or liposome

encapsulated MTP was observed in the footpad model of HSV-1 encephalitis. However, further studies using combinations of both MTP and ribavirin (see below) are now in progress.

D. Combination Therapy

Our previous observations indicated that ribavirin was effective *in vitro* (MIC_{50} = 30 ug/ml) in suppressing herpesvirus replication (data not shown), whereas *in vivo* studies (see above) indicated that it was ineffective in our treatment schedule. In addition, we have shown that MTP activates the antiviral functions of macrophages *in vitro* but a single dose given prophylactically has no effect *in vivo* in HSV-1 peneumonitis (see above). These observations led us to attempt combination therapy in which both ribavirin and MTP were given in free or liposome encapsulated form. The results summarized in Figure 18 indicate that a single dose of liposome encapsulated MTP given three days prior to challenge together with L-RIB on the day of challenge was highly effective in preventing death. Note that other combinations given on the day of or several days after virus challenge were ineffective. Likewise, administration of liposome encapsulated MTP several days prior to the administration of ribavirin in mice infected with influenza was more effective than treatment with either ribavirin or MTP alone (data not shown). These observations suggest that MTP and ribavirin act in either a synergistic or additive fashion, and provide a sound rationale for combination therapy in which both antivirals and immunostimulants are simultaneously delivered to a defined site of infection.

V. CONCLUSIONS

The data generated in the first year of this study has resulted in the following conclusions:

1. Stable, negatively charged liposomes (MLVs) containing either ribavirin or MTP can be produced in quantities required for the treatment of virus infected laboratory animals. Liposome encapsulated drugs are retained in the lung and spleen better than free drugs after intravenous administration. Both pulmonary macrophages and splenic macrophages appear to play significant roles in retention of liposome encapsulated drugs.

2. Liposome encapsulated MTP was more effective than free MTP in activating pulmonary macrophages and in enhancing protection to HSV-1 induced pneumonitis. Similarly, liposome encapsulated ribavirin was more effective than free ribavirin in the treatment of lethal influenza virus infections.

3. Liposomes may provide a distinctive therapeutic advantage when combinations of antivirals and/or immunostimulants are used in therapy.

4. The selective tissue localization and retention characteristics provided by liposome vehicles may improve the therapeutic index of a variety of antivirals used in the treatment of pulmonary infections.

VI. Recommendations

The second year of our study (Phase III; July 1985-July 1986) will examine the usefulness of the therapeutic protocols described above in the treatment of arenavirus infections. Our plan will be to use the Pichinde MHA hamster model in our initial trials of chemotherapy with liposome encapsulated ribavirin and MTP either given alone or in combination. We will select therapeutic protocols based on our reported observations with herpes and influenza during the previous year. Data obtained from the hamster model will be used to determine treatment protocols for use in the Pichinde guinea pig (strain 13) model. Particular attention will be focused on the role of activated hepatic and pulmonary immunity in resistance to and recovery from lethal virus challenge.

Finally, we have been encouraged by the results of

combination therapies in which both ribavirin and MTP were effectively used together in the treatment of herpes and influenza virus induced pneumonitis. Preliminary results indicate that combination therapy results in a synergistic or additive effect in herpes pneumonitis. Further studies are planned to evaluate the role of an activated immune system in the presence of a virustatic agent such as ribavirin. Refinement of therapeutic protocols utilizing the combined benefits of both agents may result in highly significant improvements in chemotherapeutic strategies.

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APPENDIX

TABLE 1

EFFICIENCY OF ^{14}C-RIBAVIRIN ENCAPSULATION IN LIPOSOMES

SPECIMEN	VOLUME	CPM(0.025cc)	% EXCLUDED
PBS-RIBAVIRIN (PRE-ENCAPSULATION)	3.3 cc	680000	--
SUPERNATANTS FROM:			
FIRST LIPOSOME WASH	2.1 cc	381333	36
SECOND WASH	6.2 cc	142075	39
THIRD WASH	4.8 cc	13036	3
24 HOUR WASH	3.3 cc	1755	< 1
		TOTAL	78

LIPOSOME ENCAPSULATION EFFICIENCY = 100 - 78% = 22%

TABLE 2

Antibody Response to Influenza Virus
in Ribavirin Survivors *

Mouse #	HI Titer†	Virus Challenge‡
1	512	R
2	512	R
3	512	R
4	128	R
5	256	R
6	256	R

* - Mice were given intravenous administration of liposome encapsulated ribavirin (3mg/mouse/dose) on the day of intranasal virus challenge (10 LD_{50} of influenza) and two days after infection.

† - Mice were bled on day 21 and the sera assayed for hemagglutination inhibition (HI) antibody.

‡ - Mice were rechallenged two days after bleeding with 100 LD_{50} of influenza virus; R = Resistant.

TABLE 3

Antibody Response to HSV-1 Infection
in MTP Treated Survivors

Serum Neutralization Titers*

	Free MTP† (N=17)	Liposome Encapsulated MTP‡ (N=20)
≤ 30	9 21 26	-
≤ 90	41 42 50 90	45 70
≤ 270	155 180 180	100 130 225
≤ 810	542 810	620 623 678 704 704 784 784 810
≤ 2430	1200 1400 1134 1215 1215	1134 1134 1296 1296 2430 2430
	$\bar{x}$=489 s=538	P=.09§ $\bar{x}$=840 s=668

* Mice were bled 21 days following intranasal challenge with 10 LD_{50} of HSV-1 and the sera assayed for neutralizing antibody in a 50% plaque reduction assay (PRN_{50}).

† Free MTP 100µg/mouse/dose D-3, D0 and D+2.

‡ Liposome encapsulated MTP 100µg/mouse/dose D-3, D0 and D+2.

§ Statistical significance determined by analysis of variance.

VIREMIA[c] AFTER MTP TREATMENT

TREATMENT GROUPS	HOURS POST INFECTION[a] 24	48
PBS	—[d]	5
SHAM	—	10
MTP LIP[b]	—	—
FREE MTP[b]	—	—

a - INTRANASAL CHALLENGE WITH 10 LD HSV-1
b - INTRAVENOUS INOCULATION WITH 100μg/MOUSE
3 DAYS BEFORE AND ON THE DAY OF CHALLENGE
c - PLAQUE FORMING UNITS PER MILLILITER OF BLOOD
d - NO PLAQUES

TABLE 4

FIGURE 1

FLOW CYTOMETRIC ANALYSIS OF THE SIZE DISTRIBUTION OF SHAM AND MTP LIPOSOMES

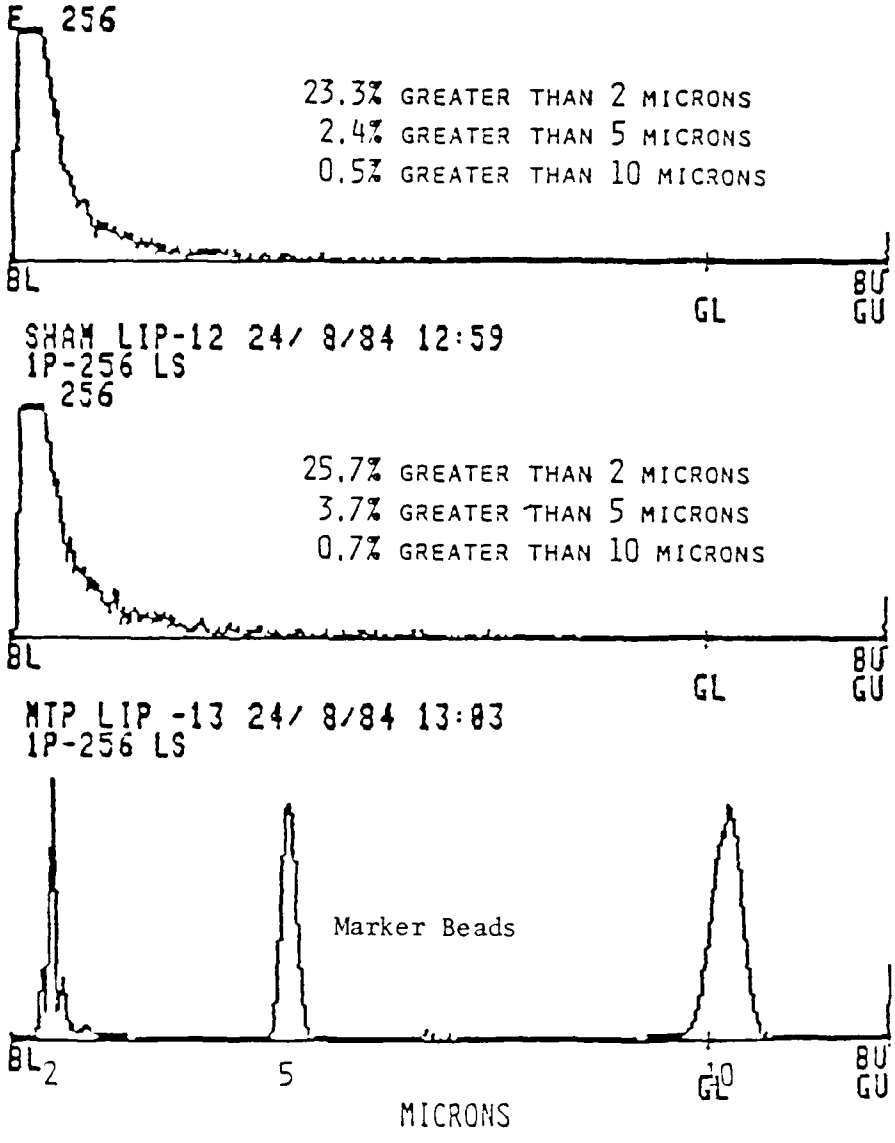


FIGURE 2

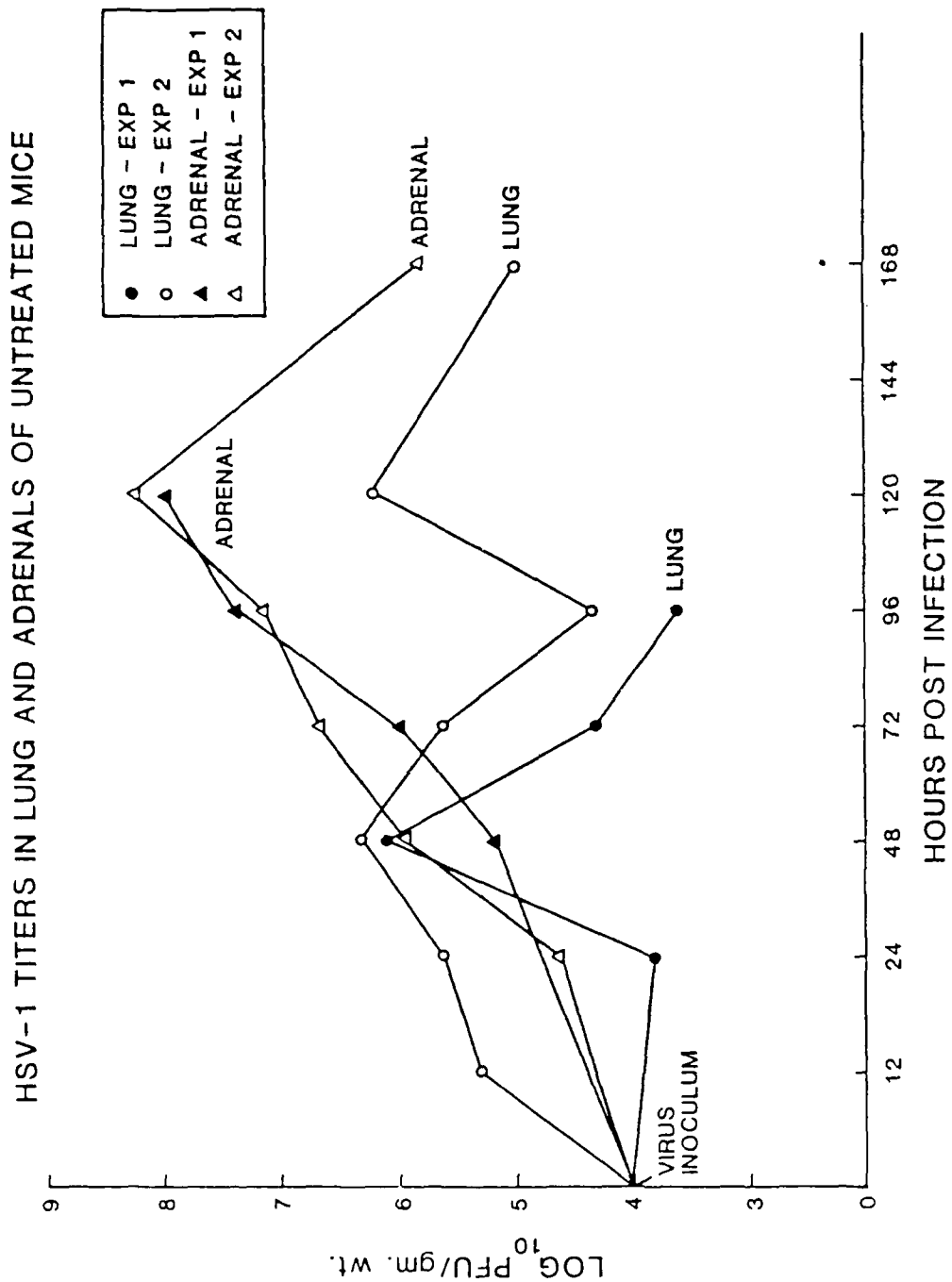

HSV-1 TITERS IN LUNG AND ADRENALS OF UNTREATED MICE
LUNG - EXP 1
LUNG - EXP 2
ADRENAL - EXP 1
ADRENAL - EXP 2
ADRENAL
LUNG
ADRENAL
LUNG
VIRUS INOCULUM
LOG10 PFU/gm. wt.
HOURS POST INFECTION
9
8
7
6
5
4
3
0
12
24
48
72
96
120
144
168

FIGURE 3

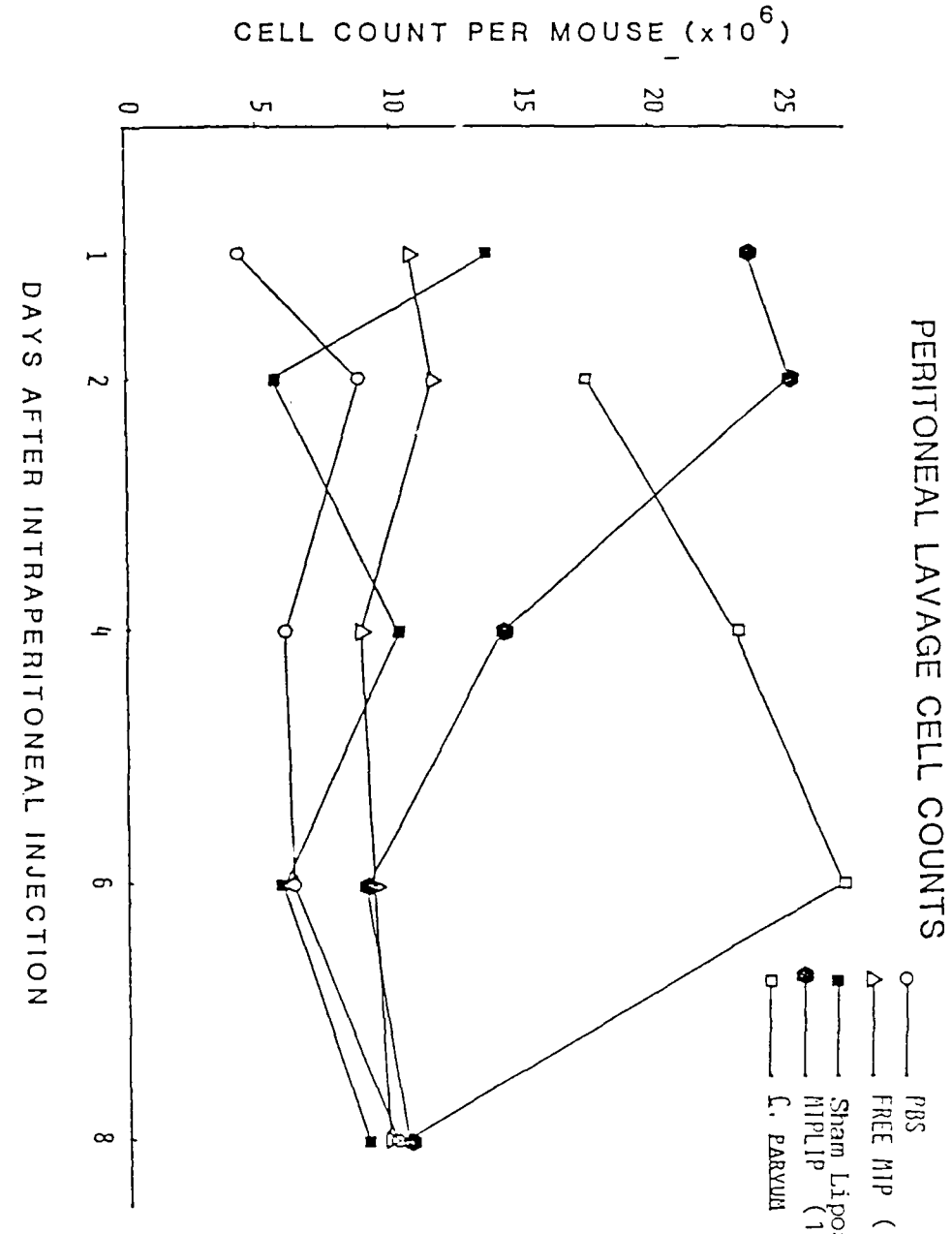
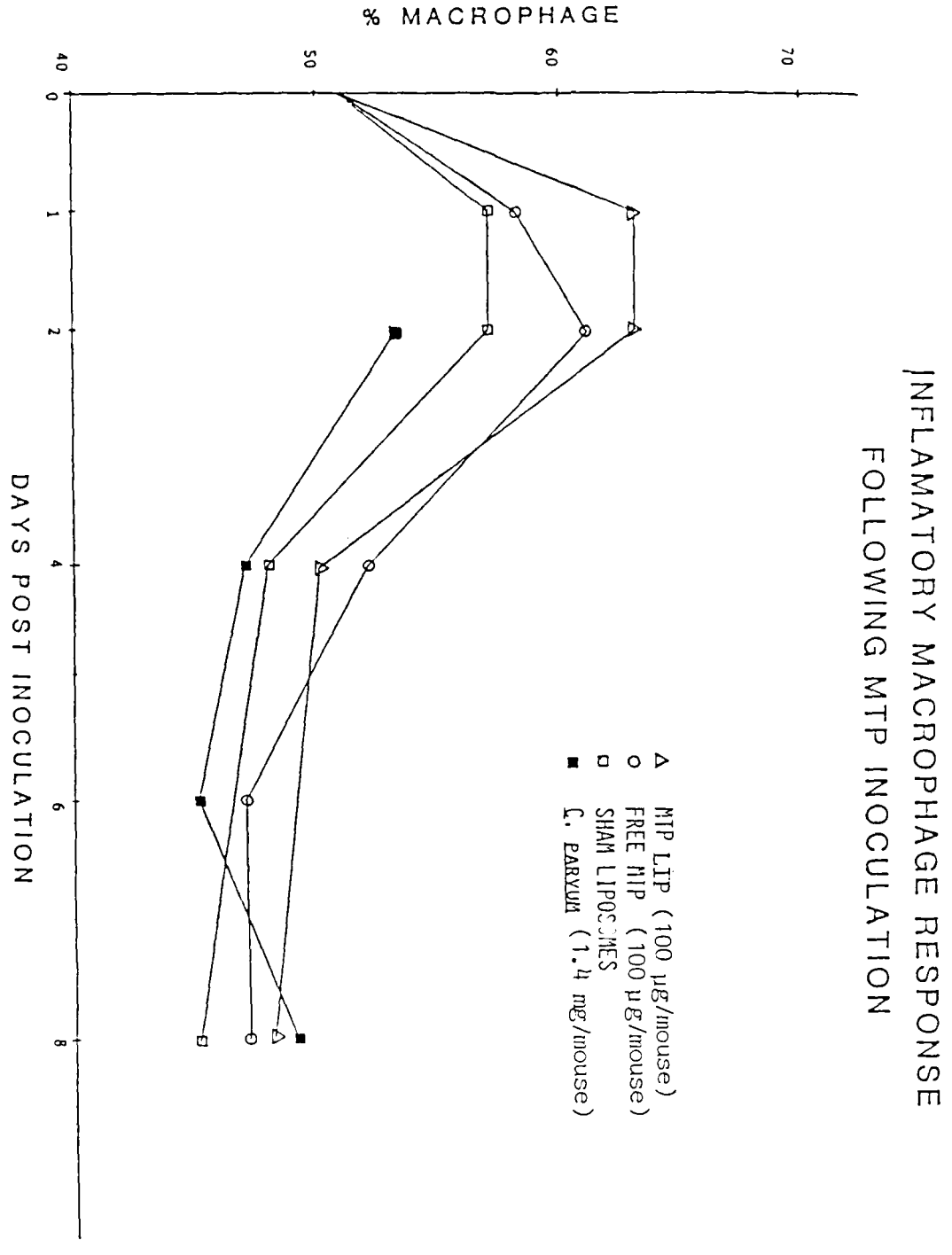
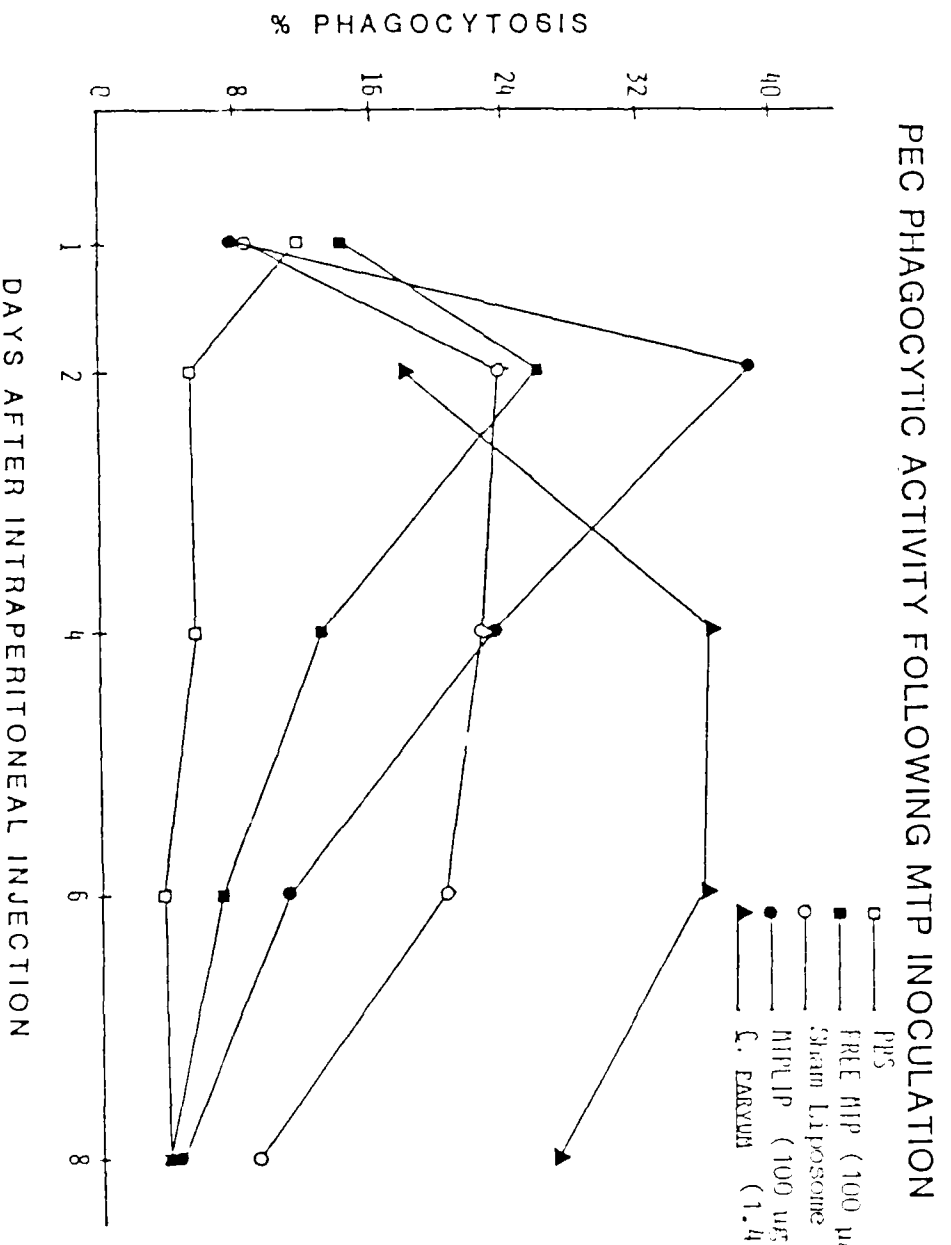

PERITONEAL LAVAGE CELL COUNTS
CELL COUNT PER MOUSE (x10^6)
0
5
10
15
20
25
1
2
4
6
8
DAYS AFTER INTRAPERITONEAL INJECTION
PBS
FREE MIP (100 µg/mouse)
Sham Liposome
MIPLIP (100 µg/mouse)
C. PARVUM (1.4 mg/mouse)

FIGURE 4



INFLAMATORY MACROPHAGE RESPONSE
FOLLOWING MTP INOCULATION

FIGURE 5


PEC PHAGOCYTIC ACTIVITY FOLLOWING MTP INOCULATION
% PHAGOCYTOSIS
0
8
16
24
32
40
1
2
4
6
8
DAYS AFTER INTRAPERITONEAL INJECTION
PBS
FREE MTP (100 µg/mouse)
Sham Liposome
MTP-LIP (100 ug/mouse)
C. PARVUM (1.4 mg/mouse)

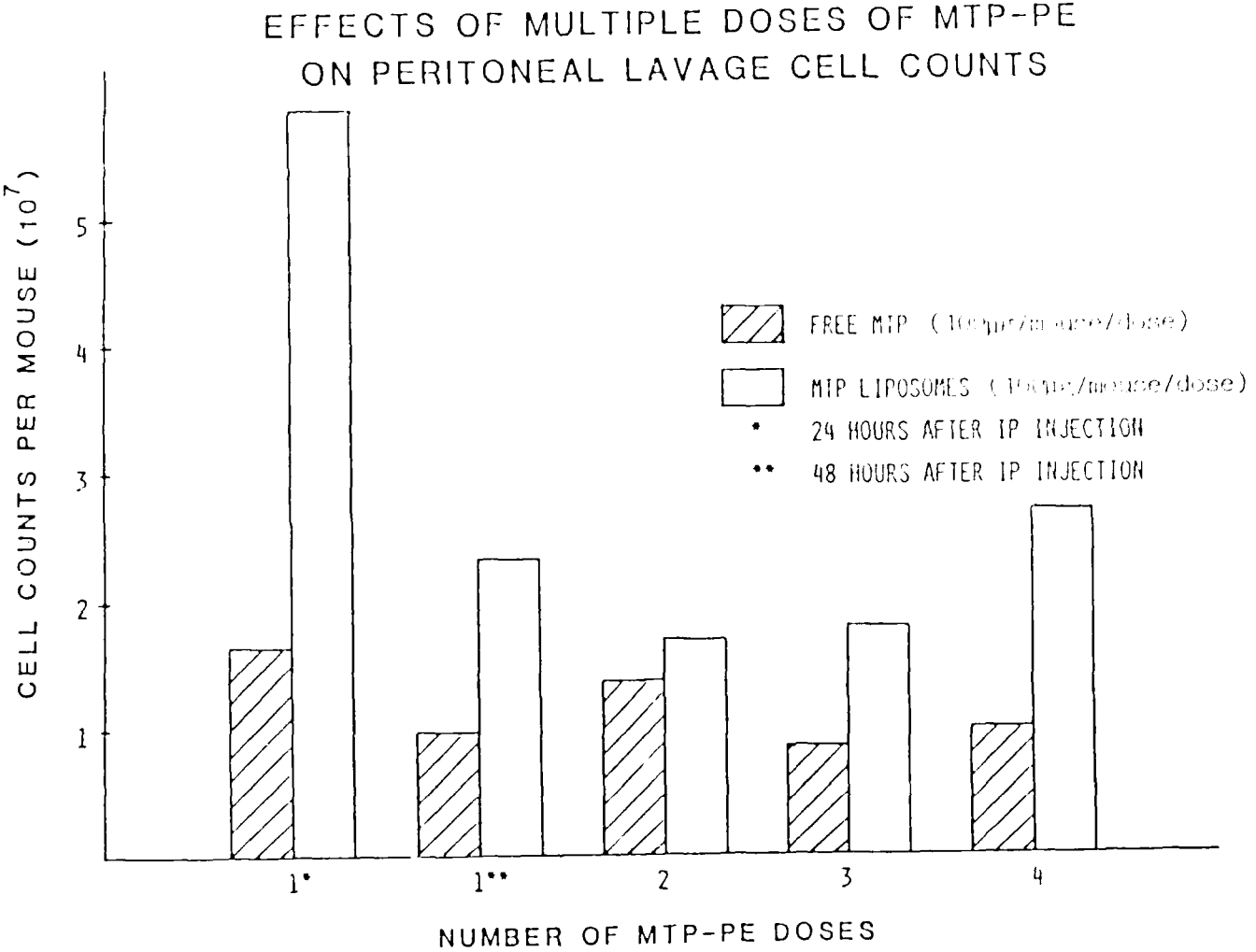

EFFECTS OF MULTIPLE DOSES OF MTP-PE
ON PERITONEAL LAVAGE CELL COUNTS
CELL COUNTS PER MOUSE (10^7)
5
4
3
2
1
FREE MTP (100μg/mouse/dose)
MTP LIPOSOMES (100μg/mouse/dose)
* 24 HOURS AFTER IP INJECTION
** 48 HOURS AFTER IP INJECTION
1*
1**
2
3
4
NUMBER OF MTP-PE DOSES

FIGURE 6

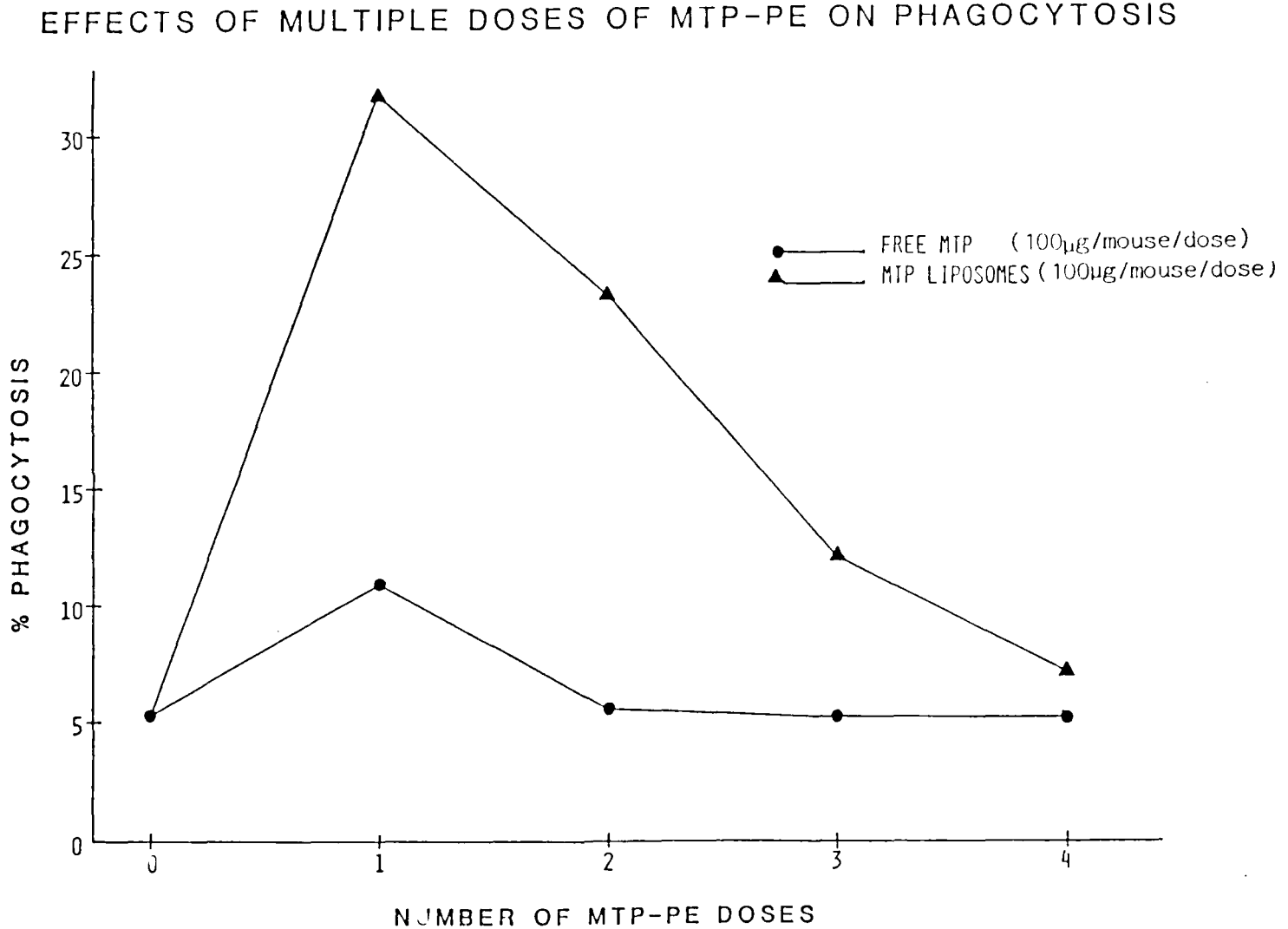

EFFECTS OF MULTIPLE DOSES OF MTP-PE ON PHAGOCYTOSIS
% PHAGOCYTOSIS
0
5
10
15
20
25
30
0
1
2
3
4
NUMBER OF MTP-PE DOSES
FREE MTP (100µg/mouse/dose)
MTP LIPOSOMES (100µg/mouse/dose)

FIGURE 7

FIGURE 8

CELL SORTER ANALYSIS OF ALVEOLAR MACROPHAGES FOLLOWING PHAGOCYTOSIS OF FLUORESCEIN LABELED S. AUREUS

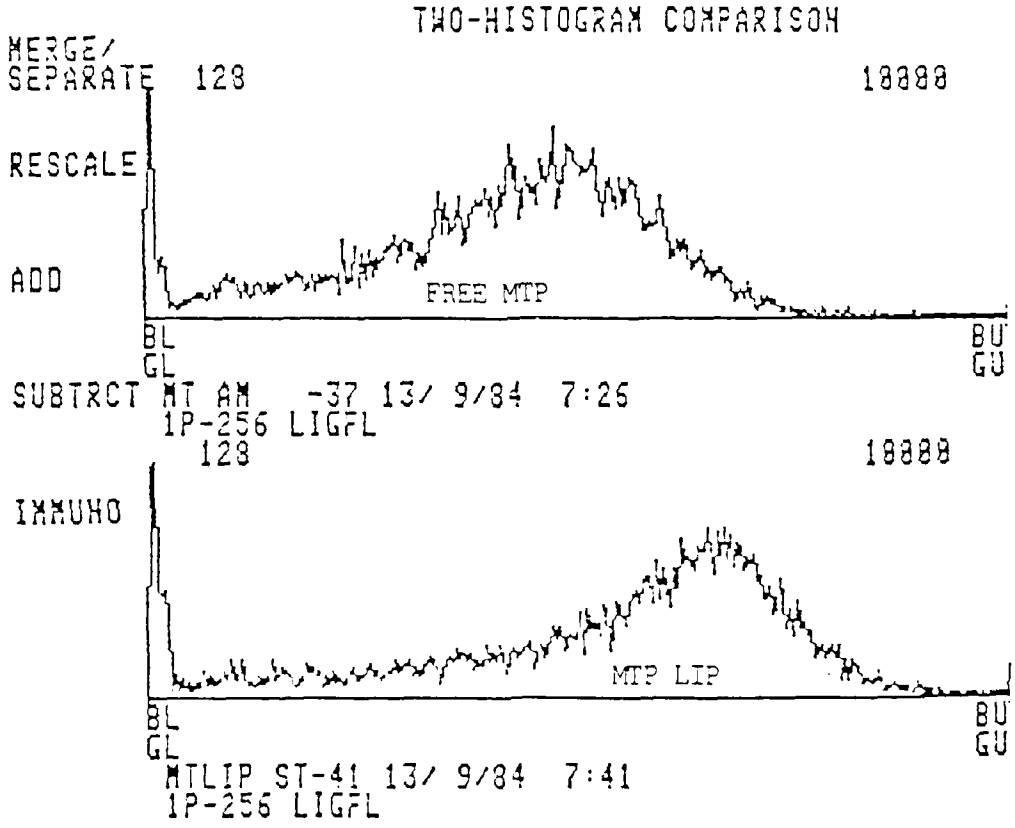


Figure 8 - Phagocytic Activity of Alveolar Macrophages Following MTP Treatment. Three groups of mice were inoculated intravenously with 0.2 ml of either sham liposome, liposome encapsulated MTP (100 ug/mouse), or free MTP (100 ug/mouse) 48 hrs prior to the instillation of 10^8 fluorescein labeled Staphylococcus aureus organisms. Lungs were transtracheally lavaged one hour after S. aureus administration and examined in an Epics IV Flow cytometer. The histograms represent the average effect observed from three mice in each group.

FIGURE 9

CELL SORTER ANALYSIS OF ALVEOLAR MACROPHAGES FOLLOWING PHAGOCYTOSIS OF FLUORESCEIN LABELED S. AUREUS

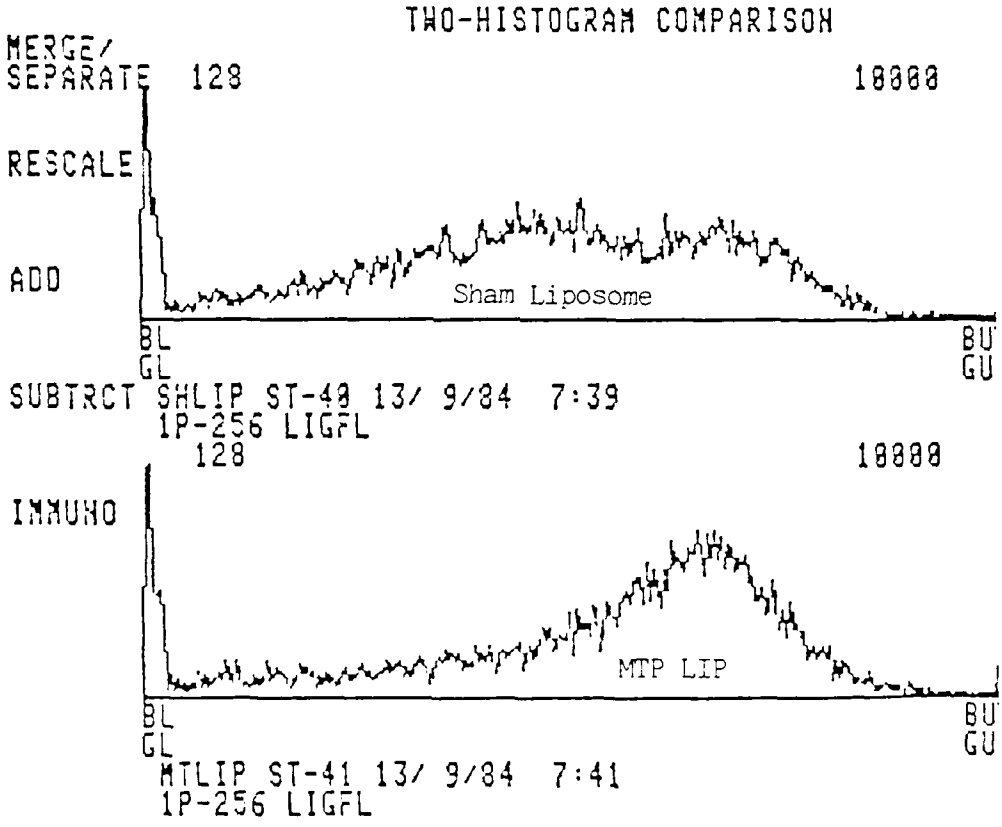
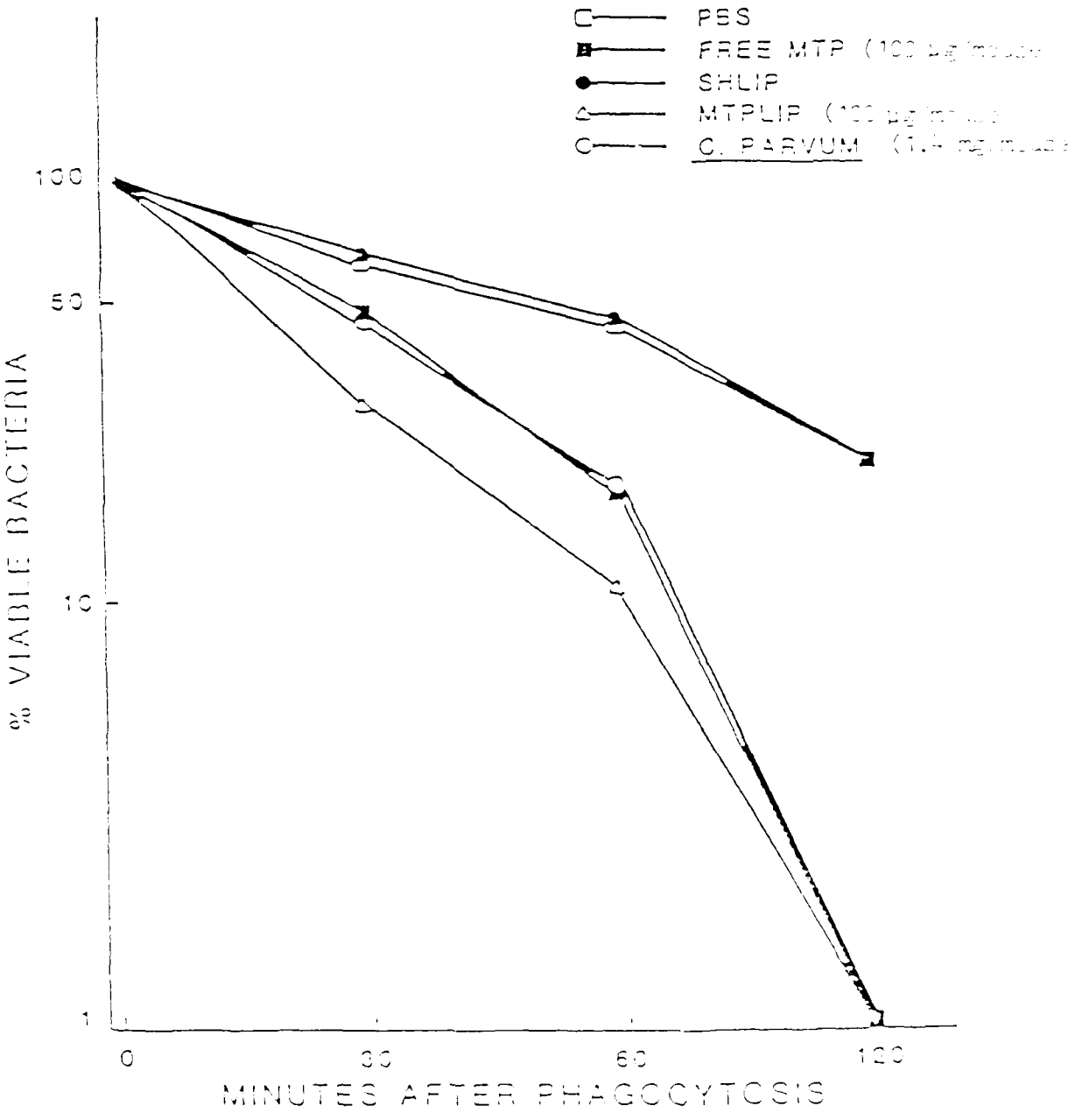


Figure 9 - Phagocytic Activity of Alveolar Macrophages Following MTP Treatment. See Figure 8 legend for details.

Figure 10 - Microbicidal Activity of Peritoneal Macrophages Following MTP Treatment. Mice were inoculated intraperitoneally with 0.2 ml of sterile, endotoxin free PBS, free MTP (100 ug/mouse), sham liposome, liposome encapsulated MTP (100 ug/mouse), or C. parvum (1.4 mg/mouse). Peritoneal macrophages from each treatment group (except C. parvum) were collected 48 after inoculation. C. parvum PEC were collected 4 days post inoculation. Washed PEC were adjusted to 5×10^6 cells/ml and incubated with 10^8 viable S. aureus organisms. Viable organisms remaining at selected time intervals were determined by plating on agar plates. The percentage of viable bacteria recorded at the indicated times represent the average obtained from three mice.

FIGURE 10



*Macrophages were collected 48 hrs. following inoculation with immunostimulant and mixed with viable S. aureus organisms for the time periods indicated.

Figures 11 and 12. Tissue Distribution of C^{14} Labeled Ribavirin.

A. Non-encapsulated (free) ribavirin.

B. Liposome-encapsulated ribavirin. Four groups of 6, 8 weeks old mice were inoculated intravenously with radiolabeled free or liposome-encapsulated ribavirin. Three mice from each time group were sacrificed by cervical dislocation and a blood sample taken prior to exsanguination. Livers, spleens, lungs and kidneys were removed, washed, and dried prior to weighing. Weighed samples were digested with tissue solubilizer and aliquots counted in a liquid scintillation counter.

FIGURE 11

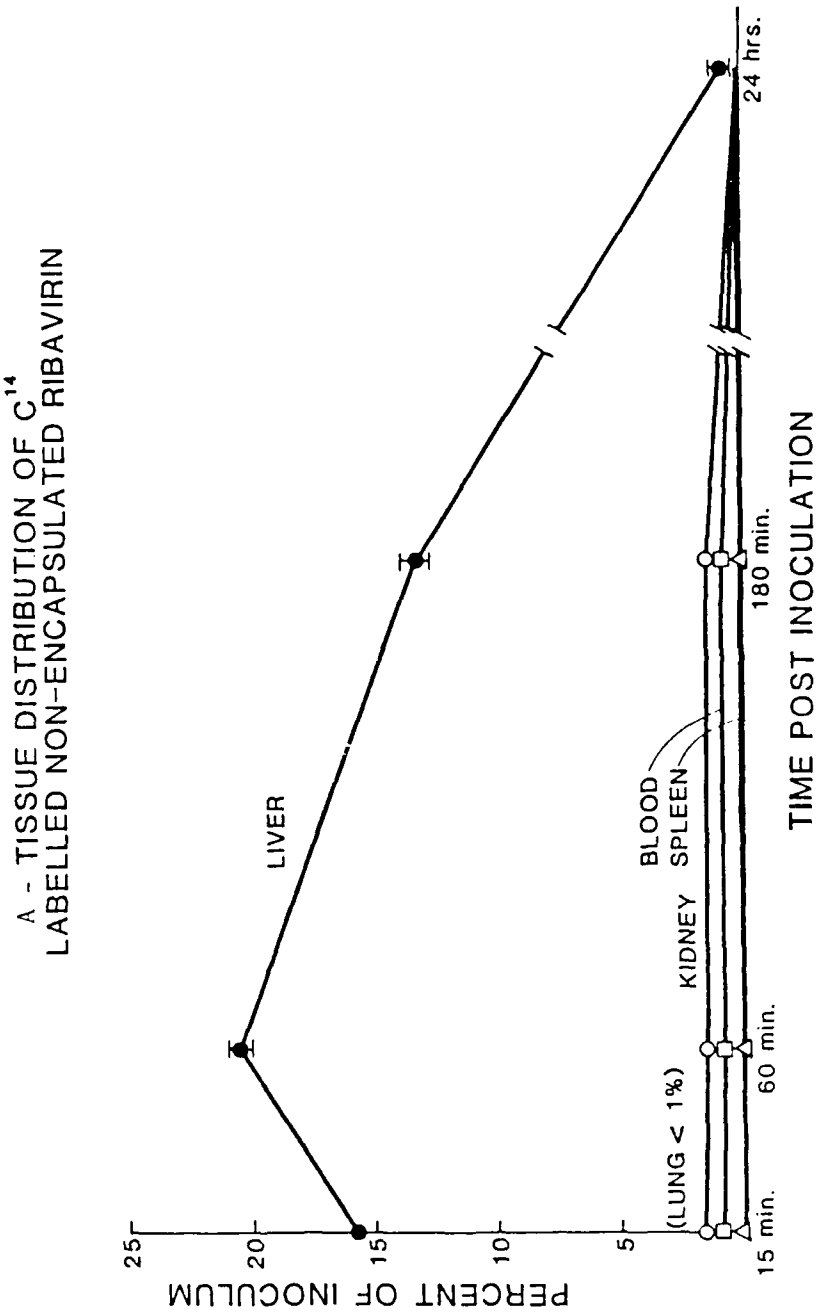
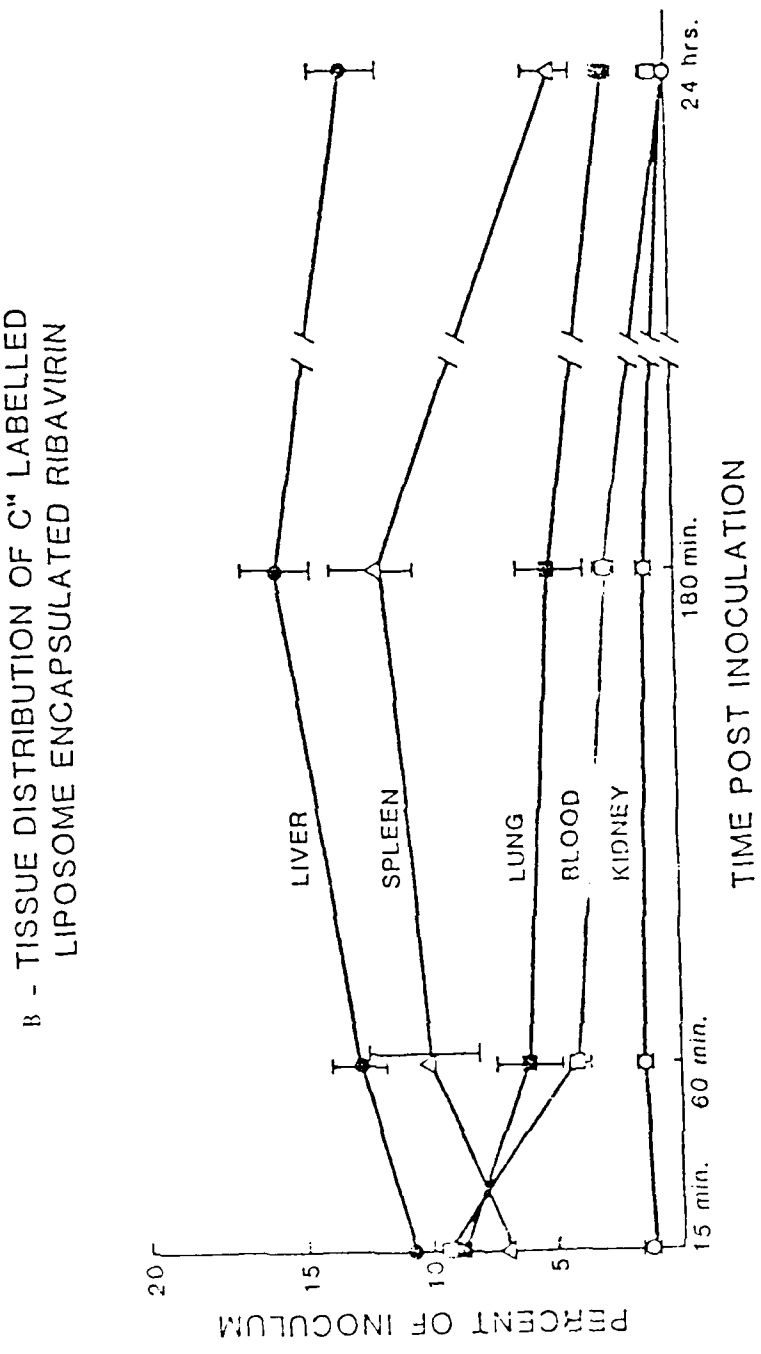

A - TISSUE DISTRIBUTION OF C^{14}
LABELLED NON-ENCAPSULATED RIBAVIRIN
PERCENT OF INOCULUM
25
20
15
10
5
LIVER
BLOOD
SPLEEN
KIDNEY
(LUNG < 1%)
15 min.
60 min.
180 min.
24 hrs.
TIME POST INOCULATION

FIGURE 12


B - TISSUE DISTRIBUTION OF C" LABELLED
LIPOSOME ENCAPSULATED RIBAVIRIN
PERCENT OF INOCULUM
20
15
10
5
LIVER
SPLEEN
LUNG
BLOOD
KIDNEY
15 min.
60 min.
180 min.
24 hrs.
TIME POST INOCULATION

RIBAVIRIN TREATMENT OF INFLUENZA VIRUS INFECTED MICE

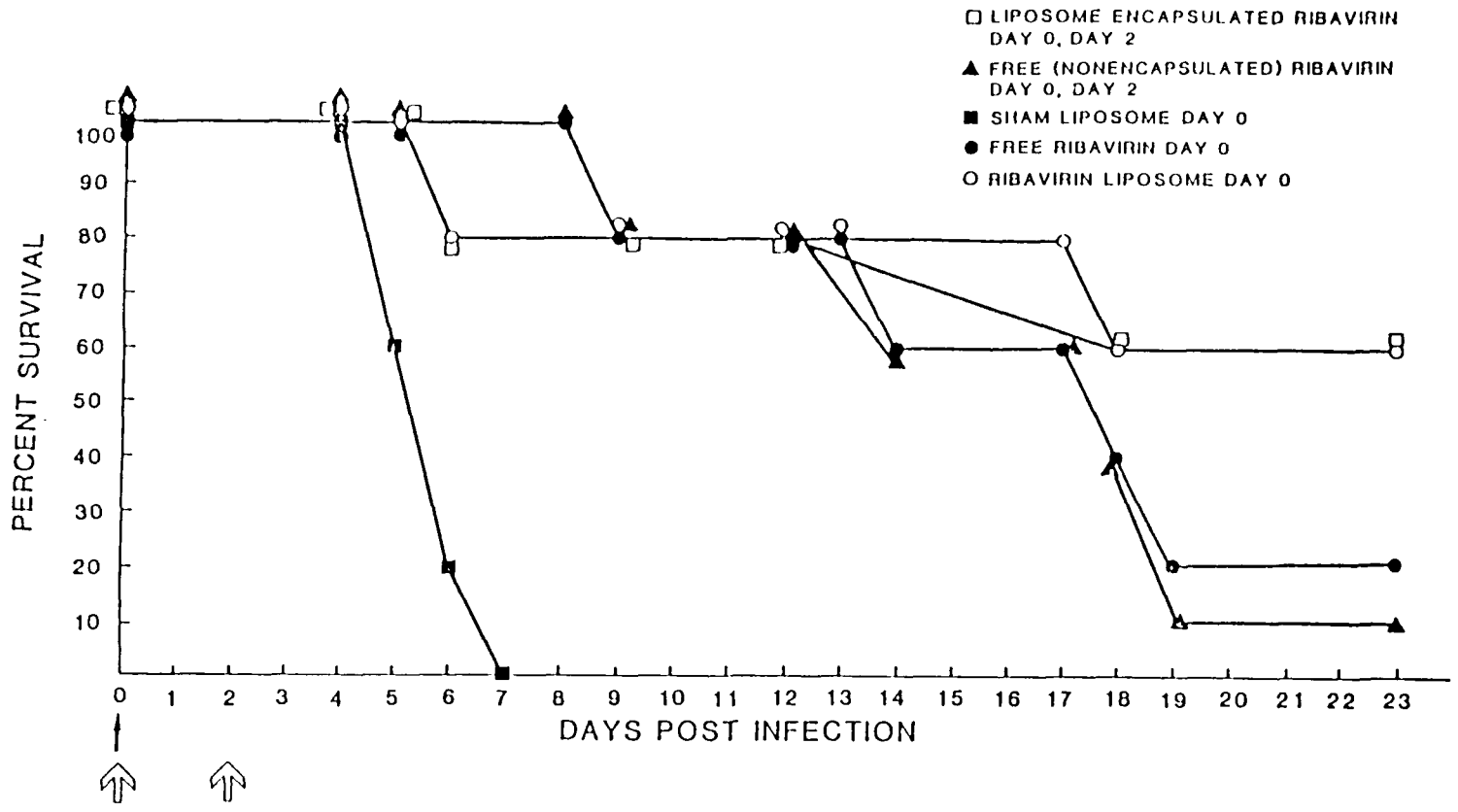


FIGURE 13

Figure 13 - Ribavirin Treatment of Influenza Virus Infected Mice.
Mice were injected intranasally with $10LD_{50}$ of influenza and treated intraveneously with either free (10 mg/mouse) or liposome encapsulated (3 mg/mouse) ribavirin on the indicated days. Each group consisted of 10 mice. Arrows indicate inoculation days.

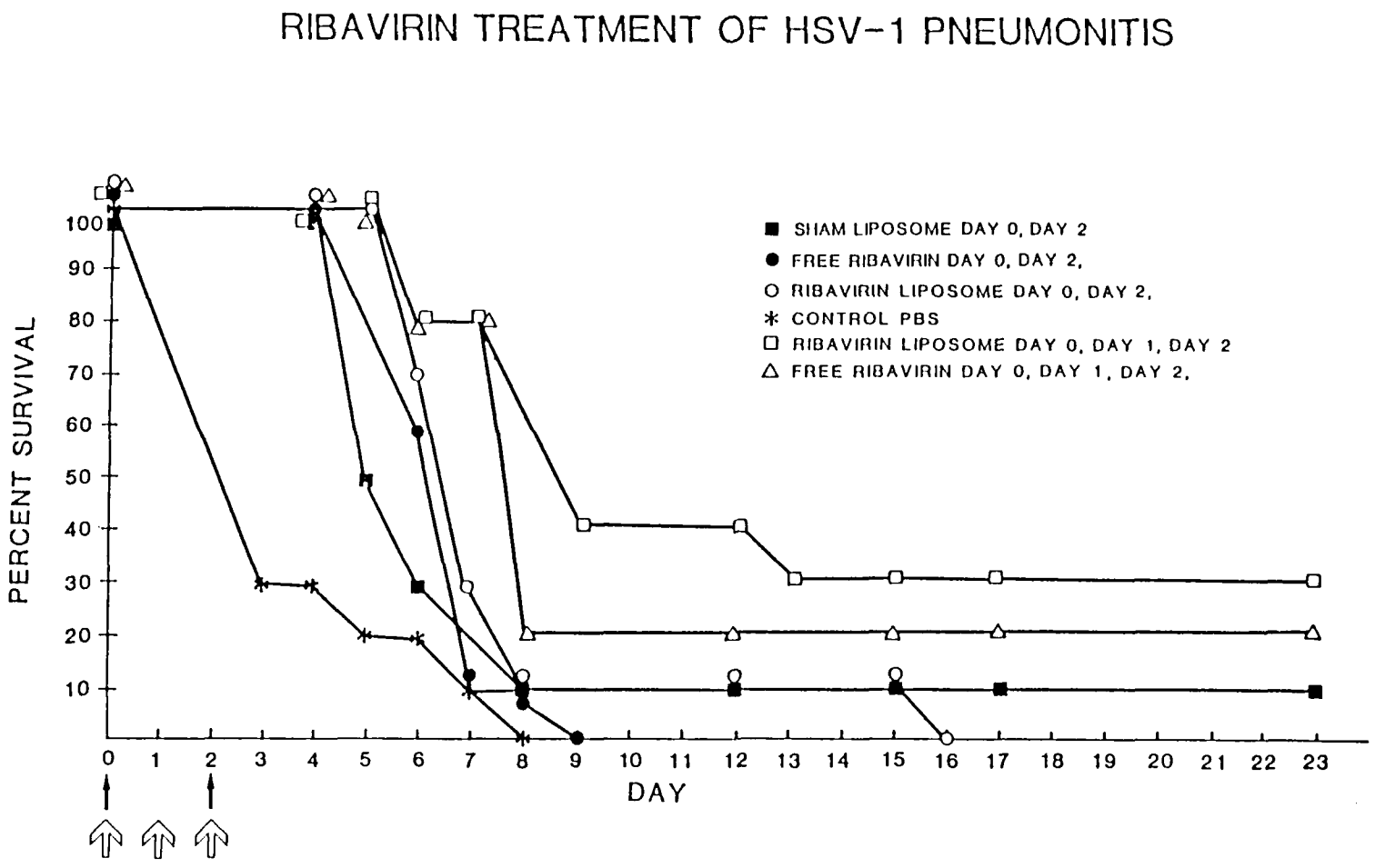


FIGURE 14

Figure 14 - Ribavirin Treatment of HSV-1 Pneumonitis. Mice were injected intranasally with $10LD_{50}$ of HSV-1 and treated with free (3 mg/mouse) or liposome encapsulated ribavirin (10 mg/mouse) on the days indicated. Each group consisted of 10 mice and were examined daily for 30 days. Arrows indicate inoculation days.

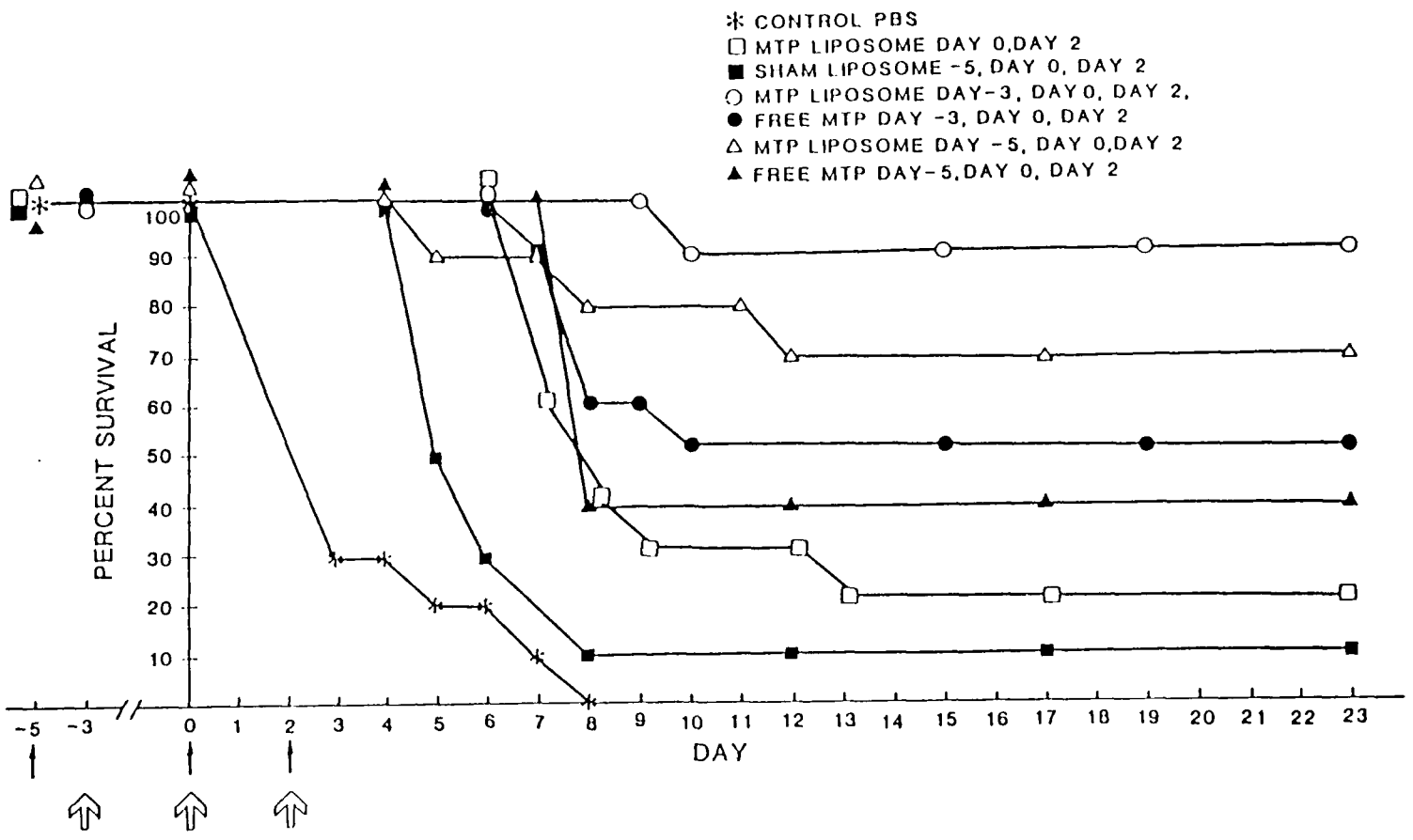


FIGURE 15

Figure 15 - MTP Treatment of HSV-1 Pneumonitis. Four-five week old mice were inoculated intravenously with 0.2 ml of either free or liposome encapsulated MTP (100ug/dose) at the times indicated and challenged intranasally with $10LD_{50}$ of HSV-1. Each group consisted of 10 mice and were examined daily for 30 days. Arrows indicate inoculation days.

FIGURE 16

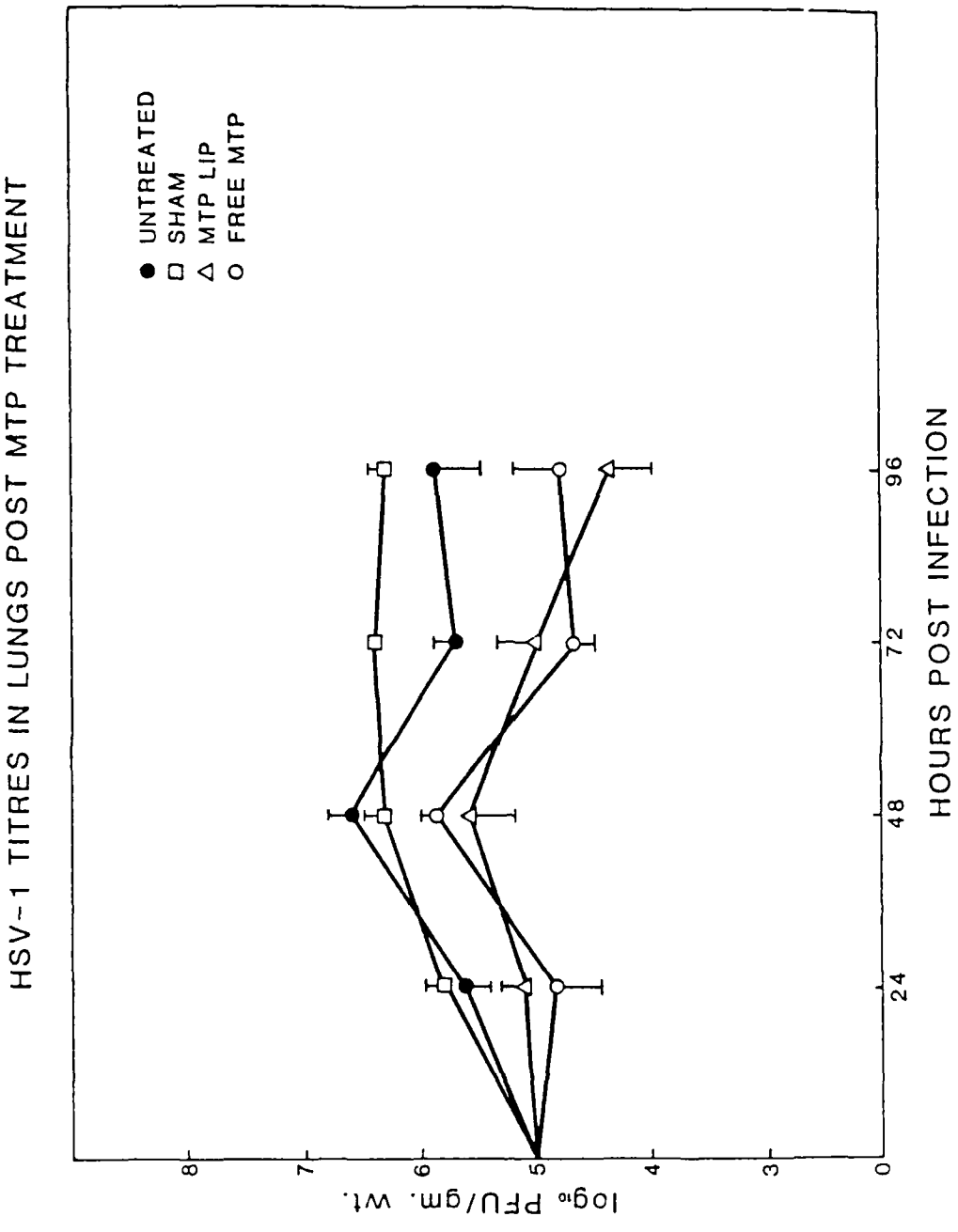

HSV-1 TITRES IN LUNGS POST MTP TREATMENT
● UNTREATED
□ SHAM
△ MTP LIP
○ FREE MTP
log₁₀ PFU/gm. wt.
0
3
4
5
6
7
8
24
48
72
96
HOURS POST INFECTION

FIGURE 17

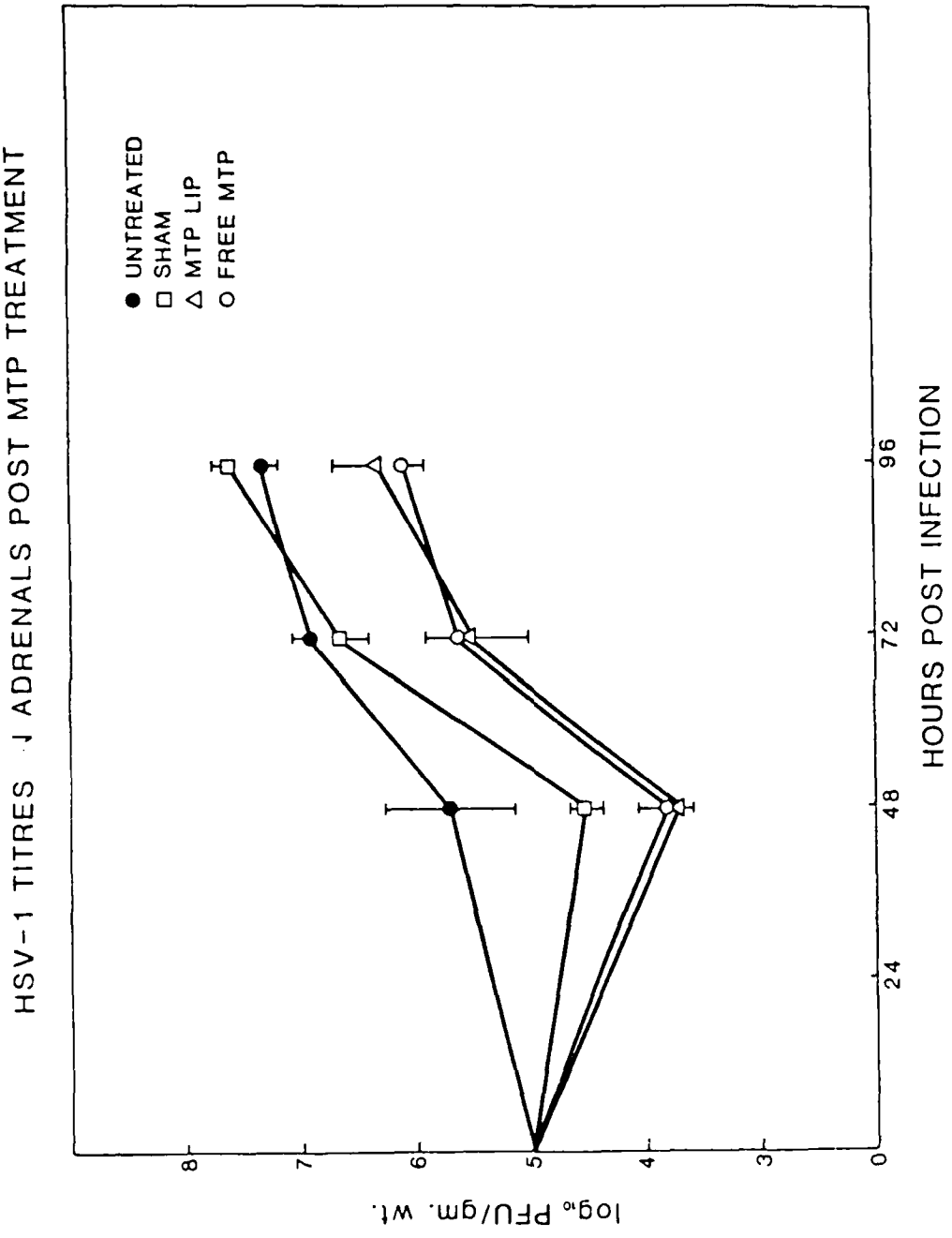
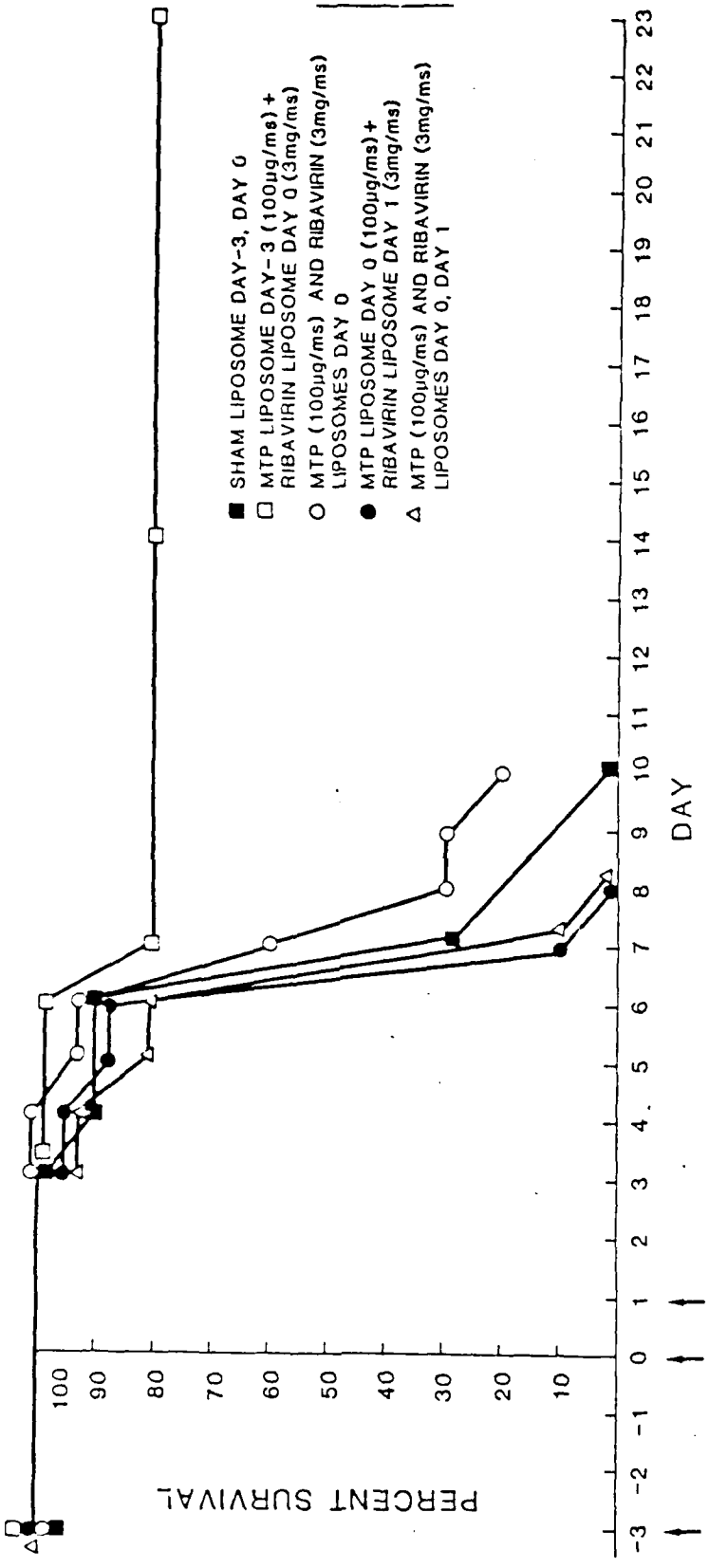

HSV-1 TITRES ⅂ ADRENALS POST MTP TREATMENT
● UNTREATED
□ SHAM
△ MTP LIP
○ FREE MTP
log₁₀ PFU/gm. wt.
8
7
6
5
4
3
0
24
48
72
96
HOURS POST INFECTION

FIGURE 18


COMBINED MTP/RIBAVIRIN THERAPY OF HSV-I PNEUMONITIS
PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
DAY
SHAM LIPOSOME DAY-3, DAY 0
MTP LIPOSOME DAY-3 (100µg/ms) + RIBAVIRIN LIPOSOME DAY 0 (3mg/ms)
MTP (100µg/ms) AND RIBAVIRIN (3mg/ms) LIPOSOMES DAY 0
MTP LIPOSOME DAY 0 (100µg/ms) + RIBAVIRIN LIPOSOME DAY 1 (3mg/ms)
MTP (100µg/ms) AND RIBAVIRIN (3mg/ms) LIPOSOMES DAY 0, DAY 1

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